ANTIBODY RECOGNITION OF THE TYPE 14 PNEUMOCOCCAL CAPSULE

Evidence for a Conformational Epitope in a Neutral Polysaccharide

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Antibody binding to polysaccharide antigens has been thought to be based upon recognition of a relatively simple oligosaccharide epitope consisting of no more than six or seven sugar residues (1-3). The existence of more complex conformational epitopes has been postulated, but there has been little experimental evidence for conformational immunodeterminants in polysaccharide antigens. Recently, however, we have found evidence of a conformational epitope recognized by antibodies directed against the capsular polysaccharide of type III group B *Streptococcus*, and others have reported similar findings with both polyclonal and monoclonal antibodies against the $\alpha(2\rightarrow8)$ linked sialic acid polysaccharide capsules of *Neisseria meningitidis* group B and *Escherichia coli* K1 (4-6). In each case, the affinity of antibody binding to derivative oligosaccharides increased as the chain length of the antigen increased, up to hundreds of sugar residues in the case of the group B streptococcal polysaccharide and up to at least 16 residues for the group B meningococcal/*E. coli* K1 system, supporting the hypothesis that anticapsular antibodies recognized a conformational epitope fully expressed only in high molecular weight forms of the saccharide.

Although the evidence for the existence of conformational epitopes in these two polysaccharides is convincing, it is not clear whether the phenomenon is relatively unique to these antigens, or whether it may be quite general among complex polysaccharide antigens. Both polysaccharide antigens for which conformational epitopes have been well described contain negatively charged sialic acid residues as a prominent feature of their repeating unit structures, as the exclusive sugar in the group B meningococcal/*E. coli* K1 polysaccharide and as side chain termini in the type III group B streptococcal polysaccharide. In addition, a mAb against the former polysaccharide crossreacts with certain other negatively charged polymeric molecules (5, 7). These observations raise the question whether the presence of negatively charged functional groups is an essential structural feature for polysaccharides to express a conformational epitope.

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The capsular polysaccharide of type 14 *Streptococcus pneumoniae* (Pn14) is structurally related to that of type III group B *Streptococcus*, differing only in that the Pn14 polysaccharide lacks sialic acid residues. This structural difference has been shown to be important in the antigenic distinctness of the two polysaccharides (8). To determine whether negatively charged functional groups are a requisite feature for polysaccharides to express a conformational epitope, we studied the characteristics of anticapsular antibody binding to oligosaccharides derived from the Pn14 polysaccharide. Our data support the existence of a conformational epitope in the Pn14 polysaccharide, despite the absence of negatively charged residues, and suggest that recognition of conformational epitopes may be a more general phenomenon than previously appreciated in the interaction of antipolysaccharide antibodies with bacterial capsular polysaccharide antigens.

Materials and Methods

Preparation of Oligosaccharides. Pn14 capsular polysaccharide was a gift from Dr. Philip Vella (Merck and Co., Inc., West Point, PA). To estimate the molecular size of the native Pn14 polysaccharide, a 5-mg sample of this material was chromatographed on a 2.6 × 90 cm column of Sepharose CL-4B in 0.3 M ammonium carbonate buffer at a flow rate of 60 ml/h, and K_{av} was calculated as described previously (9). Endo-β-galactosidase was purified from culture supernatants of *Cytophaga keratolytica* (formerly, *Flavobacterium keratolyticus*), as described previously (10). Oligosaccharides were produced by incubating 5–20 mg of Pn14 polysaccharide with endo-β-galactosidase in 10 mM sodium acetate, 2 mM calcium chloride, pH 7.0, at 37°C for 2–4 d. Oligosaccharides consisting of one repeating unit (tetrasaccharide) and two repeating units (octasaccharide) were purified from the digestion mixture on a 1.6 × 84 cm column of Sephadex G25 (Pharmacia Fine Chemicals, Piscataway, NJ). The mixture was loaded on the column in a volume of 2.5 ml and eluted with deionized water at a flow rate of 16 ml/h. Elution of oligosaccharides was monitored by measuring ultraviolet absorbance at 206 nm (Lambda 3A Spectrophotometer, Perkin Elmer Corp., Oak Brook, IL). The fractions (2.6 ml) were assayed by thin-layer chromatography (TLC), as described below, and fractions containing one or two repeating unit oligosaccharides were pooled separately. The pooled fractions were lyophilized and rechromatographed on the same column, if necessary, to yield preparations that gave a single oligosaccharide band on TLC. A pool of larger molecular size oligosaccharides was isolated by fractionation on a column of Sephadex G75 (Pharmacia Fine Chemicals). For this preparation, 0.5 g solid ammonium sulfate was added per milliliter of incubation mixture after digestion to precipitate the enzyme, which was removed by centrifugation. The oligosaccharide mixture was then loaded onto a 1.6 × 88 cm column of Sephadex G75 and eluted with deionized water at a flow rate of 20 ml/h. Elution of oligosaccharides was monitored by measuring ultraviolet absorbance at 206 nm. Molecular size of oligosaccharides was estimated by calculation of K_{av} based on dextran standards of 10,000 and 20,000 average M_r. Fractions (2.8 ml) containing oligosaccharides of estimated M_r of 12,000 to 22,000 were pooled and lyophilized as the "22 repeating unit pool."

Thin-Layer Chromatography. One and two repeating unit oligosaccharides were identified and their purity was confirmed by TLC. Samples containing 2–10 μg of carbohydrate in a volume of 3–8 μl were applied to 10 × 10 cm plastic backed TLC plates of silica gel 60 (E. Merck, Darmstadt, FRG). Plates were developed for 75–90 min in a solvent system of 1-butanol/acetic acid/water, 2:1:1, then dried and stained with diphenylamine spray (11).

Gas-Liquid Chromatography (GC). Native Pn14 polysaccharide and the purified single repeating unit oligosaccharide were subjected to acid hydrolysis by treatment with 0.5 M trifluoroacetic acid at 100°C for 16 h. Component monosaccharides were reduced and peracetylated by the method of Sawardeker (12), and analyzed by GC. Samples were analyzed on a Hewlitt-Packard 5880A instrument equipped with a flame ionization detector, using a 25 m × 0.25 mm column of fused silica polycyanopropylsiloxane, 0.2 microns (Silar 10C; Alltech Associates, Deerfield, IL) with a temperature program from 230° to 250°C.

Preparation of IgG and Fab Fragments. Rabbit antiserum prepared by the Statenseruminstitut (Copenhagen) against type 14 *S. pneumoniae* was obtained through Dako Corp. (Santa Barbara, CA). 1 ml of serum was loaded onto a 1 × 5 cm column of Protein A coupled to Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO). After washing with 35 ml of PBS, pH 7.3, bound IgG was eluted with 0.1 M glycine/HCl, pH 3.0. Fractions were neutralized with 0.1 M sodium hydroxide. Fab fragments were prepared from protein A-purified IgG by papain cleavage using papain linked to agarose beads (Sigma Chemical Co.), as described (13). Fc fragments were removed by adsorption to protein A-Sepharose, as described above. Absence of residual intact IgG in the Fab preparation was confirmed by SDS-PAGE.

SDS-PAGE. Samples of IgG and Fab fragments were subjected to SDS-PAGE under reducing conditions, as described by Laemmli (14). Protein bands were detected by staining with Coomassie blue (Bio-Rad Laboratories, Richmond, CA).

ELISA Inhibition Assay. Native Pn14 polysaccharide was coupled to poly-L-lysine (Sigma Chemical Co.) by the method of Gray (15) and coated onto 96-well flat-bottomed plastic plates (Dynatech Laboratories, Alexandria, VA) at a concentration of 2 μg/ml polysaccharide in 40 mM sodium phosphate buffer, pH 7.0, at 4°C overnight. After washing three times with 40 mM sodium phosphate, pH 7.0, containing 0.05% Tween-20 (Sigma Chemical Co.), nonspecific protein binding sites were blocked by incubating wells with 200 μl of 5% skim milk powder (Difco Laboratories, Detroit, MI) in the same buffer (PBT) at 37°C for 1 h. After washing three times with PBT, anti-Pn14 IgG or Fab fragments (diluted 1:2,000 or 1:1,600, respectively, in PBT) were added, and incubated at 37°C for 1 h. For inhibition assays, the inhibiting polysaccharide or oligosaccharide was premixed with the IgG or Fab preparation immediately before addition to the ELISA wells. After washing three times with the same buffer, 200 μl of alkaline phosphatase-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) diluted 1:3,000 in PBT was added and incubated at 37°C for 1 h. After washing three times, *p*-nitrophenyl phosphate (Sigma Chemical Co.) was added at a concentration of 1 mg/ml in 40 mM sodium carbonate buffer, pH 9.6, containing 0.01% magnesium chloride. After incubation at 37°C for 30–60 min, A_{405} was determined using an ELISA reader (model EL-307; Bio-Tek, Burlington, VT).

Results

Preparation of Oligosaccharides from Pn14 Capsular Polysaccharide. The Pn14 capsular polysaccharide has a linear backbone with a trisaccharide repeating unit structure; galactosyl residues are linked as monosaccharide side chains to the *N*-acetyl-glucosamine residues of each backbone repeating unit as shown in Fig. 1 (16). Because of the branched structure of the polysaccharide, a nonspecific method of depolymerization such as acid hydrolysis would not only result in random cleavage of the backbone, but also loss of side chain residues. Instead, we used a highly specific endo-glycosidase to digest the polysaccharide in order to generate oligosaccharide fragments that preserved the native repeating unit structure, including the branch galactosyl residues. Endo-β-galactosidase, purified from culture supernatants of *Cytophaga keratolytica*, catalyzes the hydrolysis of galactose β(1→4) glucose bonds of

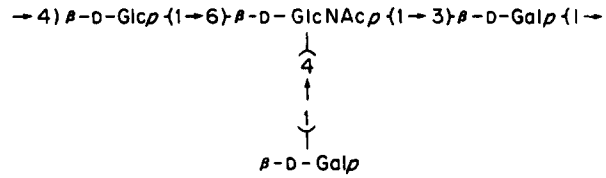

FIGURE 1. Repeating unit structure of type 14 pneumococcus capsular polysaccharide.

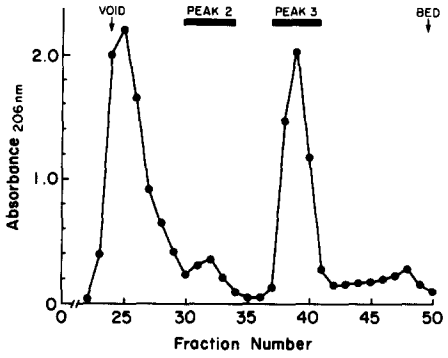

FIGURE 2. Sephadex G25 elution profile of oligosaccharides produced by endo-β-galactosidase digestion of Pn14 polysaccharide. Column fractions in peak 3 were pooled and lyophilized to yield the 1 repeating unit oligosaccharide. Fractions in peak 2 were pooled, lyophilized, and rechromatographed on the same column. Fractions in peak 2 of the second column run were pooled and lyophilized to yield the 2 repeating unit oligosaccharide.

susceptible polysaccharides, including the Pn14 capsular polysaccharide that contains one such linkage per backbone repeating unit. Complete digestion of the polysaccharide yields the tetrasaccharide single repeating unit oligosaccharide of the structure shown in Fig. 1, while partial digestion yields fragments consisting of two or more repeating units. After enzymatic digestion of the Pn14 polysaccharide, one repeating unit (tetrasaccharide) and two repeating unit (octasaccharide) oligosaccharides were purified from the digestion mixture by gel filtration chromatography on Sephadex G25. Oligosaccharides eluted from the column in three major peaks, as assessed by UV absorbance at 206 nm (Fig. 2). Peak 3 contained an oligosaccharide with migration characteristics on TLC compatible with that expected for the single repeating unit tetrasaccharide (Fig. 3, lane *5*). Component glycose analysis of the purified single repeating unit oligosaccharide by GC demonstrated that the oligosaccharide contained the same molar ratios of individual sugars as the native Pn14 polysaccharide, confirming that the oligosaccharide contained a complete repeating unit (Table I). Peak 2 contained, as a predominant species, an oligosaccharide with TLC mobility consistent with its identification as the two repeating unit octasaccharide. The column fractions comprising Peak 2 were pooled, lyophilized, and rechromatographed on the same column to yield the purified preparation of 2 repeating unit oligosaccharide shown in Fig. 3, lane *4*.

A pool of higher molecular weight oligosaccharides was purified from the digestion mixture by chromatography on Sephadex G75. The column was calibrated with dextran standards, and fractions with elution volumes corresponding to that expected

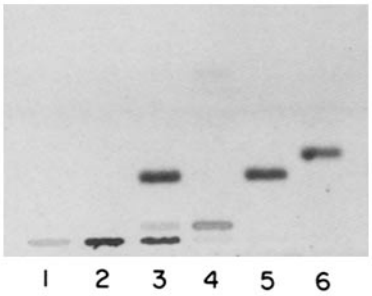

FIGURE 3. TLC of oligosaccharides produced by endo-β-galactosidase digestion of Pn14 polysaccharide. (Lane *1*) endo-β-galactosidase; (lane *2*) Pn 14 native polysaccharide; (lane *3*) endo-β-galactosidase plus Pn14 polysaccharide, after digestion; (lane *4*) Sephadex G25 purified 2 repeating unit oligosaccharide; (lane *5*) Sephadex G25 purified 1 repeating unit oligosaccharide; (lane *6*) stachyose standard.

TABLE I
Component Sugar Analysis of Pn14 Polysaccharide and 1 Repeating Unit Oligosaccharide

Glycose*	Molar ratios‡	
	Pn14 native polysaccharide	Pn14 1 repeating unit oligosaccharide
D-galactose	1.98 (2)	2.03 (2)
D-glucose	1.00 (1)	1.00 (1)
N-acetyl-D-glucosamine	0.751 (1)	0.742 (1)

* Analyzed as alditol acetates.
‡ Values are means of duplicate determinations. Nearest integer ratios are shown in parentheses.

for oligosaccharides of 12,000 to 22,000 M_r were pooled (Fig. 4). This pool contained oligosaccharides having an estimated average molecular size of ~17,000 M_r, or 22 repeating units, with a range of 15 to 28 repeating units.

Purification of IgG and Fab Fragments from Immune Rabbit Serum. IgG was purified from immune rabbit serum on a column containing Protein A coupled to agarose beads. The purified IgG preparation was digested with papain to generate Fab fragments. Fc fragments and any residual intact IgG were removed from the Fab preparation by adsorption to protein A-agarose. Analysis of the IgG and Fab preparations by SDS-PAGE showed the absence of a major band of ~58,000 M_r in the Fab preparation, as expected for loss of IgG heavy chains, as well as a new or enriched band of ~28,000 M_r, as expected for the Fab heavy chain cleavage fragment (Fig. 5).

Inhibition of IgG or Fab Binding to Pn14 Polysaccharide by Derivative Oligosaccharides. The relative affinity of antibody binding to saccharides of different chain lengths was assessed by an ELISA inhibition assay. IgG purified from immune rabbit serum was premixed with varying concentrations of inhibiting oligo- or polysaccharide, then added to microtiter wells previously coated with native Pn14 polysaccharide. Alkaline phosphatase-conjugated anti-rabbit IgG was added, followed by substrate, and the amount of inhibition measured by decrement in optical density compared with control wells to which no inhibitor was added. Inhibition curves were gener-

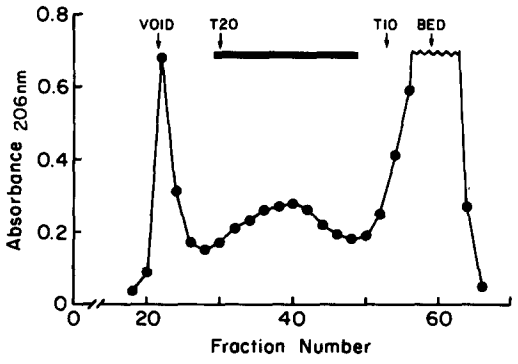

FIGURE 4. Sephadex G75 elution profile of oligosaccharides produced by endo-β-galactosidase digestion of Pn14 polysaccharide. T10 and T20 indicate elution volumes of dextrans of average M_r of 10,000 and 20,000, respectively. Bar indicates column fractions that were pooled to yield the 22 repeating unit oligosaccharide pool.

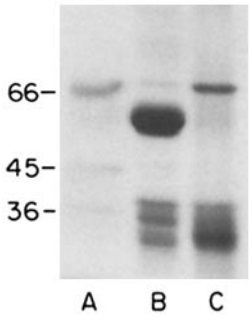

FIGURE 5. SDS-PAGE of IgG and Fab fragments prepared from anti-Pn14 rabbit serum. (*A*) molecular size markers; (*B*) IgG preparation; (*C*) Fab preparation.

ated using oligosaccharides of 1 repeating unit, 2 repeating units, 22 repeating units, and the native Pn14 polysaccharide. Each increment in molecular size of the inhibiting antigen was associated with a shift to the left in the resultant inhibition curve (Fig. 6 *A*). The concentration of inhibitor required to achieve 50% inhibition decreased progressively with increasing saccharide chain length from 5.6×10^{-4} M for the single repeating unit oligosaccharide to 7.0×10^{-11} M for the native polysaccharide (Table II). Inhibition curves were also generated using the same inhibiting oligosaccharides, with Fab fragments as the primary antibody instead of intact IgG (Fig. 6 *B*). The relative affinities of Fab binding to the inhibiting antigens were very

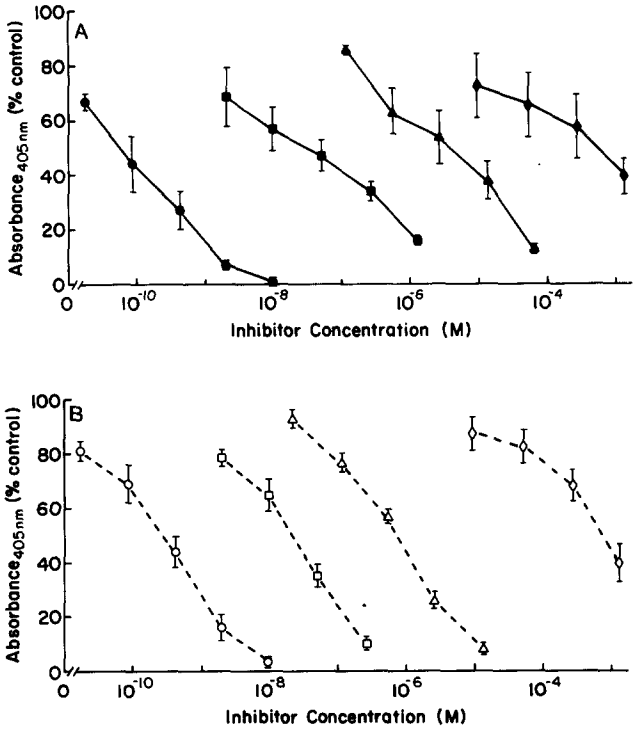

FIGURE 6. Inhibition of Pn14 ELISA by oligosaccharides of different chain lengths. (*A*) inhibition of anti-Pn14 IgG binding by oligosaccharides of 1 repeating unit (*diamonds*), 2 repeating units (*triangles*), 22 repeating units (*squares*), and the native Pn14 polysaccharide (*circles*). (*B*) inhibition of anti-Pn14 Fab fragment binding by oligosaccharides of 1 repeating unit (*diamonds*); 2 repeating units (*triangles*), 22 repeating units (*squares*), and the native Pn14 polysaccharide (*circles*).

TABLE II
Concentrations of Oligosaccharides Producing 50% Inhibition of Antibody Binding in Native Pn14 ELISA

Inhibiting antigen	Molecular size	50% inhibitory concentration	
		IgG	Fab
	M_r	M	
1 Repeating unit oligosaccharide	761	5.6×10^{-4}	8.0×10^{-4}
2 Repeating unit oligosaccharide	1,522	4.0×10^{-6}	7.6×10^{-7}
22 Repeating unit oligosaccharide	17,000*	3.0×10^{-8}	2.2×10^{-8}
Native Pn14 polysaccharide	2,000,000‡	7.0×10^{-11}	2.7×10^{-10}

* Estimated by K_{av} on Sephadex G75.
‡ Estimated by K_{av} on Sepharose CL-4B.

similar to those observed for intact IgG, ranging from 8.0×10^{-4} M for Fab binding to the single repeating unit oligosaccharide to 2.7×10^{-10} M for Fab binding to the native polysaccharide (Table II). For the purposes of these experiments, the molecular size of the native Pn14 polysaccharide was estimated to be 2,000,000 M_r or 2,500 repeating units, based on its K_{av} of 0.2 on a column of Sepharose 4B-CL. Note that this is a conservative estimate: if the true molecular size were larger, the inhibition curves for the native polysaccharide with both IgG and Fab fragments would be shifted further to the left.

Discussion

Conformational epitopes have been described previously for the capsular polysaccharides of type III group B *Streptococcus* and group B meningococcus/*E. coli* K1 (4, 5). These antigens have in common the presence of sialic acid residues, as the exclusive sugar in the group B meningococcus/*E. coli* K1 capsule, and as side chain termini in the group B streptococcal capsular antigen (10, 17). A human mAb that recognizes a conformational determinant in the group B meningococcus/*E. coli* K1 polysaccharide also recognizes the polynucleotides poly(A) and poly(I), suggesting that the polyanionic nature of the antigen was a critical feature of the conformational epitope (5, 7). Similarly, the charged sialic acid residues of the type III group B streptococcal polysaccharide have been shown to influence the conformation of that polysaccharide and to play an important role in the immunodeterminant (10, 18). That the negatively charged sialic acid residues of each of these polysaccharides appeared to play such a central role in their conformational epitopes suggested that expression of such an epitope might be uniquely associated with acidic polysaccharides. The capsular polysaccharide of type 14 pneumococcus represents the third bacterial polysaccharide antigen for which a conformational epitope has been described, and the first example of such an epitope in a neutral polysaccharide. However, for the Pn14 polysaccharide, we present clear evidence for a conformational epitope that does not depend upon the presence of negatively charged residues.

Oligosaccharide fragments consisting of one or more complete tetrasaccharide repeating units were derived from the capsular polysaccharide of type 14 pneumococcus by specific enzymatic hydrolysis of backbone galactose $\beta(1\rightarrow4)$ glucose bonds. ELISA inhibition experiments demonstrated a progressive increase in the affinity of anticap-

sular antibody binding to these oligosaccharide antigens, as saccharide chain length increased from 1 to 2, from 2 to 22, and from 22 to 2,500 repeating units. The increase in antibody binding affinity between the one repeating unit tetrasaccharide and two repeating unit octasaccharide may be explained by more complete filling of the Fab combining site by the latter antigen. However, abundant experimental evidence indicates that the Fab cleft of antipolysaccharide antibodies is optimally filled by an epitope corresponding to no more than six or seven monosaccharide residues (1–3). Therefore, the further increases in affinity observed for antibody binding to the 22 repeating unit oligosaccharide and to the native polysaccharide indicate that the antibody recognizes a conformational determinant that is fully expressed only in high molecular weight forms of the antigen. That small oligosaccharides of the same repeating structure are able to completely inhibit antibody binding to the native polysaccharide at high oligosaccharide concentrations indicates that the same antibodies bind to the smaller antigens as to the native polysaccharide, but bind with lower affinity. These results suggest that, in the polymeric form of the antigen, the antibody binding epitope is stabilized in a particular conformation that antibody recognizes. The conformation of the same epitope in oligomeric forms of the antigen is less favorable for antibody binding, resulting in a lower affinity interaction. Similar results were obtained using intact IgG and isolated Fab fragments, suggesting that recognition of the conformational epitope is a property intrinsic to the Fab antigen combining region, and does not depend on divalent Ig binding or Fc-mediated cooperative interactions between antibody molecules.

Much of our current understanding of antibody recognition of polysaccharide antigens is based on studies of model systems using linear homopolymers of a single sugar (1–3). The conformation of a linear homopolymer of a single sugar linked by a single type of glycosidic bond is determined by the type of glycosidic linkage present, which controls the position of each sugar ring relative to the adjacent residues to which it is linked. The repetitive nature of the linkage pattern results in a regular, periodic structure of the polymer, which may be described generally as a helix. For example, a $\beta(1\rightarrow4)$ linked glucan has a regular repeating conformation described as an extended ribbon, while a $\beta(1\rightarrow3)$ linked glucan forms a hollow helix (19). It is not possible, currently, to accurately predict the conformation of more complex polysaccharides based on their primary linkage structures; however, several complex polysaccharides have been crystallized into fibers and studied by x-ray diffraction. In each case, the x-ray diffraction pattern has suggested the polysaccharide has a regular helical shape (20–22). It seems plausible, then, that antipolysaccharide antibodies may recognize a unique structure determined not only by the sequence and linkages of the sugars making up the antibody binding epitope, but also by the particular conformation imparted to the epitope by the overall shape of the polymer. It may be noteworthy that α or $\beta(1\rightarrow6)$ linked glucans, such as the dextrans originally studied by Kabat and coworkers (1, 2), have a higher degree of conformational entropy than glucans having any other bonding arrangement. Because carbon six is separated from the pyranose ring by a carbon-carbon bond, a $1\rightarrow6$ linkage permits greater mobility of adjacent sugar rings than linkage to any of the ring carbons. Therefore, $1\rightarrow6$ linked dextrans are uniquely flexible, and less likely to assume a stable conformation in solution (19). While our traditional concept of antibody recognition of polysaccharide antigens is that only six or seven sugar residues form

the binding epitope, the experimental system upon which this concept was originally based may be the exception rather than the rule in terms of conformational stability and, hence, potential for expression of a conformational epitope.

In light of the current demonstration of a conformational epitope in a third bacterial polysaccharide, it seems likely that further examples will be found as other bacterial polysaccharides are studied. The repeating unit structures of many bacterial capsular polysaccharides closely mimic the oligosaccharides present as the carbohydrate moieties of mammalian glycoproteins and glycolipids, presumably reflecting an evolutionary strategy by which parasites mask their surface antigens with capsules whose antigenic determinants resemble those of the host, taking advantage of self-tolerance to evade immune detection (23, 24). The ability of host antibodies to recognize a conformational epitope expressed only in the polymeric form of a saccharide antigen may provide a mechanism by which the host immune system recognizes a bacterial capsular polysaccharide as distinct from host oligosaccharides having a similar or identical primary repeating structure. Many bacterial polysaccharides are immunogenic in animals and human subjects, despite their structural similarity to host oligosaccharides. Conformational epitopes may explain how a specific antibody response is elicited by a polysaccharide having a repeating unit structure identical to a host oligosaccharide without producing crossreactions between the antipolysaccharide antibodies and host antigens.

Summary

Oligosaccharides consisting of one or more tetrasaccharide repeating units were derived from the capsular polysaccharide of type 14 pneumococcus (Pn14) by endo-β-galactosidase digestion. The relative affinity of anticapsular antibody binding to derivative oligosaccharides of different chain lengths was measured in a Pn 14 ELISA inhibition assay. The concentration of inhibiting antigen required to achieve 50% inhibition of IgG binding increased progressively from 5.6×10^{-4} M to 7.0×10^{-11} M as the inhibiting saccharide chain length increased from 1 tetrasaccharide repeating unit to 2,500 repeating units. These data indicate that antibodies directed against the Pn14 polysaccharide recognize a conformational epitope fully expressed only in high molecular weight forms of the antigen. Similar results were found for inhibition of Fab fragment binding, suggesting that recognition of the conformational epitope is largely dependent on the intrinsic affinity of the Fab combining region. Unlike previously reported polysaccharides for which conformational epitopes have been described, the Pn14 polysaccharide does not contain negatively charged residues, indicating that expression of conformational determinants is not limited to acidic polysaccharides. Antibody recognition of conformational epitopes may be a common mechanism by which the host immune response discriminates between bacterial polysaccharides and host oligosaccharides of similar structure.

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